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Opsonization of *Cryptococcus neoformans* by Human Immunoglobulin G: Role of Immunoglobulin G in Phagocytosis by Macrophages

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The role of immunoglobulin G (IgG) as an opsonin in phagocytosis of *Cryptococcus neoformans* by macrophages was investigated. Labeling with ^{125}I showed that IgG isolated from normal human serum bound to non-encapsulated *C. neoformans*. Furthermore, IgG-opsonized cryptococci were agglutinated by antiserum to IgG heavy chains, indicating that normal human serum contains antibody that will bind to the yeast surface. The IgG isolated from normal serum accounted for all opsonizing activity found in normal human serum, since differences were not noted between the opsonizing activities of whole serum, heat-inactivated serum and purified IgG when these opsonins were compared at equivalent concentrations of IgG. Phagocytosis of IgG-opsonized cryptococci was inhibited by anti-macrophage IgG, a reagent known to block Fc-mediated attachment and ingestion, and by pepsin digestion of opsonizing IgG. Thus, IgG opsonization is an Fc-dependent process. Opsonizing IgG appears to play its major role during the attachment phase of phagocytosis, since antimacrophage IgG blocked attachment of cryptococci to macrophages but could not block ingestion of IgG-opsonized cryptococci that had been allowed to attach to macrophages. Ingestion of opsonized cryptococci was not blocked by 2-deoxy-D-glucose, a reagent known to block Fc-mediated ingestion, thus confirming that IgG has a primary role in attachment and suggesting that ingestion is mediated by a process that is not Fc dependent.

The mechanism by which capsular polysaccharides inhibit phagocytosis is not known. Previous reports from our laboratory (8, 10, 17) have utilized the encapsulated yeast *Cryptococcus neoformans* in an attempt to further understand inhibition of phagocytosis. Encapsulated strains of *C. neoformans* are highly resistant to phagocytosis, whereas weakly encapsulated and non-encapsulated cryptococci are readily engulfed by neutrophils and macrophages (1, 4, 8, 13). Earlier studies have shown that cryptococcal polysaccharide inhibits the attachment stage of phagocytosis (10). Our studies have been directed toward a definition of the serum opsonins necessary for phagocytosis of non-encapsulated cryptococci, particularly the attachment phase of phagocytosis, since the phagocytosis-enhancing activities of these opsonins are a likely target for the phagocytosis-inhibiting action of the capsular polysaccharide.

Phagocytosis of *C. neoformans* by neutrophils and macrophages is dependent on a variety of serum opsonins. Diamond et al. (3) found that yeast opsonization for phagocytosis by neutrophils was dependent on components of the classical and alternative complement pathways. Immunoglobulin G (IgG) acted indirectly, functioning primarily to activate the alternative pathway. Heat-labile opsonins were also necessary for optimal phagocytosis of *C. neoformans* by thioglycolate-activated (17) and glycogen-activated (13) macrophages. In constrast, nonactivated macrophages require heat-stable serum opsonins for optimal phagocytosis of the yeast but have no requirement for heat-labile opsonins (17).

The identity of the heat-stable opsonin for phagocytosis of *C. neoformans* by normal macrophages is not known, but IgG is a likely possibility. Participation of macrophages in Fc-mediated phagocytosis is well known. Furthermore, there is indirect evidence that normal human serum may contain low levels of anti-cryptococcal IgG. Diamond et al. (3) were unable to detect anti-cryptococcal antibody in normal human serum by indirect immunofluorescence; however, the opsonic activity of the serum was reduced by adsorption of serum with cryptococci at 0°C and was restored by addition of IgG isolated from normal human serum. The stimulus for

this presumably naturally occurring antibody is not known. Unfortunately, direct evidence for natural antibody to *C. neoformans* is lacking, and there is no evidence that IgG can directly participate as an opsonin in phagocytosis of the yeast. The present study was undertaken (i) to provide direct evidence that normal human serum contains antibody to *C. neoformans,* (ii) to determine whether IgG is the heat stable opsonin in normal serum, and (iii) to determine the role of opsonizing IgG in the attachment and ingestion phases of phagocytosis.

MATERIALS AND METHODS

Yeast. *C. neoformans* strain 602 is a non-encapsulated isolate whose characteristics have been described elsewhere in detail (8, 9). Yeast cells used in phagocytosis assays were Formalin killed (10) and used as a suspension in Hanks balanced salt solution (HBSS; GIBCO, Grand Island, N.Y.) containing antibiotics (100 U of penicillin and 100 μg of streptomycin per ml; GIBCO) and buffered to pH 7.2 with sodium bicarbonate.

Phagocytosis assays. Unstimulated peritoneal macrophages were obtained from 6- to 8-week-old Swiss mice (Microbiological Associates, Walkersville, Md.). The procedure for collection and culture of macrophages has been described previously (10). Monolayers were prepared in four-chamber tissue culture chamber/slides (Lab Tek Products, Naperville, Ill.; model 4804) and incubated for 24 to 48 h at 37°C in 2.6% CO_2 before use. Each monolayer contained approximately 2.5×10^5 macrophages.

For phagocytosis assays, the culture medium was decanted, and each monolayer was washed two times with warm (37°C) HBSS. Suspensions of the yeast were then added in a 1-ml volume to each chamber. Yeast suspensions typically consisted of: (i) 10^6 yeast cells, (ii) a source of opsonin, and (iii) enough HBSS to give a final volume of 1 ml. Some experimental protocols required the use of yeast cells preopsonized with IgG [602(IgG)] or sheep erythrocytes preopsonized with IgG [E(IgG)]. 602(IgG) were prepared by incubating cells of strain 602 with normal human IgG for 30 min at 37°C and for 30 min at 4°C. Opsonized yeasts were sedimented and suspended to 10^7 yeast cells per ml in HBSS. Typically, 0.1 ml of 602(IgG) was added to 0.9 ml of medium on the macrophage monolayer. E(IgG) were prepared by mixing washed sheep erythrocytes (10^9/ml in phosphate-buffered saline, pH 7.2) with an equal volume of an appropriate dilution (usually 1:400 or 1:800) of purified IgG (7S) antibodies against intact sheep erythrocytes. The mixture was incubated for 30 min at 37°C and for 30 min at 4°C, centrifuged, and suspended to 10^8 cells per ml in HBSS. A 0.1-ml amount of E(IgG) was added to 0.9 ml of HBSS on the macrophage monolayer.

Phagocytosis of yeast cells was determined after incubation of monolayers with yeast cells for 1 h at 37°C. After incubation, the medium was decanted, the slides were washed two times in HBSS to remove nonadherent yeasts, and the slides were air dried. The macrophages were fixed with methanol and stained by the Giemsa procedure. Slides were examined microscopically, and 200 macrophages per monolayer were observed for attached or ingested yeasts. The percentage of macrophages with ingested yeasts (percent phagocytosis) or the mean number of ingested yeasts per macrophage (phagocytic index) was determined. Results presented are mean values from at least four monolayers.

Phagocytosis of erythrocytes was determined after incubation of monolayers with E(IgG) for 1 h at 37°C. After incubation, the medium was decanted, and the monolayers were washed for 15 s in HBSS diluted 1:5 in distilled water to lyse nonphagocytized erythrocytes. The slides were washed two times in HBSS and fixed for 10 min with 2% glutaraldehyde in phosphate-buffered saline (PBS). Slides were examined by phase-contrast microscopy, and percent phagocytosis or phagocytic indices were determined in the same manner as phagocytosis of yeast cells.

Attachment assays were done by preincubating monolayers at 4°C for 1 h before addition of 602(IgG) or E(IgG). The yeast or erythrocyte suspensions were added and incubated with macrophages at 4°C for 2 h. Engulfment was negligible at the low temperature. After attachment, slides were fixed and examined as before; however, lysis of external erythrocytes was omitted.

Phagocytosis of zymosan, Formalin-treated sheep erythrocytes, and latex particles was determined in some experiments. Zymosan (Sigma Chemical Co., St. Louis, Mo.) was washed three times with HBSS, and 7.5×10^6 particles in HBSS were added to each monolayer. Formalin-treated erythrocytes were prepared by the method of Holland et al. (6), and 1 ml of a 0.02% suspension was added to each monolayer. Latex particles (Sigma Chemical Co.) were washed three times in HBSS, and 2.0×10^7 particles in HBSS were added to each monolayer. All slides were fixed and examined in the same manner as slides used to study erythrocyte phagocytosis.

Statistical analysis of all phagocytosis data was done by analysis of variance.

Serum and serum components. Pooled normal human serum (GIBCO; lot no RO555100) was the source of opsonins for *C. neoformans.* IgG was isolated from human serum by diethylaminoethyl-cellulose chromatography (20) and was stored in PBS at −80°C. IgG isolated by this technique produced a single precipitin line in Ouchterlony diffusion and immunoelectrophoresis against anti-whole human serum (Meloy Laboratories, Inc., Springfield, Va.; lot no 88204). The concentration of IgG in human serum was assayed by radial diffusion in plates containing antiserum to human IgG (Kallestad Laboratories, Inc., Chaska, Minn.). Goat antiserum monospecific for the heavy (gamma) chains of human IgG was obtained from Meloy Laboratories, Inc. (lot no. 78077). $F(ab')_2$ fragments of normal human IgG were prepared by the pepsin digestion techniques of Spiegelberg and Weigle (16). IgG antibodies against sheep erythrocytes were obtained from Cordis Laboratories (Miami, Fla.). Each lot of serum was titrated to determine the optimal dilution for opsonization of erythrocytes.

Anti-macrophage antibodies were prepared in rabbits against unstimulated mouse macrophages by the

techniques of Holland et al. (6). The IgG fraction of rabbit anti-macrophage serum [AM(IgG)] was isolated by diethylaminoethyl-cellulose chromatography and was stored in PBS at −80°C. The AM(IgG) was cytotoxic for mouse macrophages at 625 μg/ml and inhibited phagocytosis of E(IgG) at a concentration of 125 μg/ml.

Radioiodination of IgG. Human IgG was labeled with ^{125}I by the lactoperoxidase method (11, 18) in borosilicate glass tubes (10 by 75 mm) at room temperature. Materials for radioiodination were added in the following order: 10 μl of $Na^{125}I$ (120 mCi/ml in PBS; New England Nuclear Corp. Boston, Mass.), 2.5 mg of human IgG (10 to 15 mg/ml in PBS), and 5 μl of lactoperoxidase (2 mg/ml in PBS; Calbiochem, La Jolla, Calif.) A 5-μl amount of H_2O_2 (0.88 mM) was added to initiate the reaction, and the mixture was mixed intermittently for 5 min. An additional 5 μl of H_2O_2 was added for an additional 5 min. Cold phosphate buffer was added (0.05 M potassium phosphate, pH 7.5) to bring the volume to 1.0 ml, and the sample was immediately applied to a G-25 (fine) Sephadex column (0.8 by 13.5 cm). The first radioactive peak was collected for use.

Samples were counted in a liquid scintillation counting fluor (Gammafluor Research Co., Hackensack, N.J.). Sample vials were counted for 5 to 10 min, using a variable set ^{125}I window in a Beckman LS-355 liquid scintillation counter.

Agglutination of opsonized cryptococci by antiserum to IgG. Cells of strain 602 (2.5×10^6) in 0.2 ml of PBS were incubated in tubes (10 by 75 mm) for 20 min at room temperature with various concentrations of human IgG (0.1 ml) in PBS. Goat antiserum monospecific for the heavy (gamma) chain of human IgG (0.1 ml of a 1:4 dilution) was added and incubated an additional 20 min at room temperature. The tubes were centrifuged, and agglutination was determined by macroscopic examination of sedimentation patterns and by microscopic examination for yeast agglutination.

Reagents. Crystallized bovine serum albumin was obtained from Miles Research Laboratories, Inc., Elkhart, Ind., and was radio-iodinated in the same manner as IgG. Grade III 2-deoxy-D-glucose was obtained from Sigma Chemical Co. Minimal essential medium with Earle base was obtained from K. C. Biological, Inc., Lenexa, Kans. and was buffered to pH 7.2 with sodium bicarbonate before use.

RESULTS

Binding of human IgG to *C. neoformans*. Diamond et al. (3) were unable to detect binding of human IgG to cryptococci by indirect immunofluorescence. A more sensitive technique should show binding of IgG to the yeast surface if the IgG does indeed act directly as an opsonin for phagocytosis of non-encapsulated cryptococci by macrophages. Adhesion of IgG from pooled normal IgG to strain 602 was demonstrated by the use of ^{125}I-labeled IgG. Cells of strain 602 (10^7) were incubated for 30 min at room temperature with various amounts of ^{125}I-labeled IgG in a total reaction volume of 1 ml. The cells were washed eight times with 0.05 M phosphate buffer (pH 7.5) to remove nonreactive immunoglobulins, and the amount of radioactivity remaining bound to the yeast cells was determined. The results of several experiments (Table 1) showed that approximately 0.12 to 0.35% of the IgG bound to the yeast cells. Thus, in a typical phagocytosis experiment in which 5 mg of IgG is used to opsonize 10^7 cryptococci, approximately 4×10^5 antibody molecules would bind to each yeast cell. ^{125}I-labeled bovine serum albumin was used as a control for nonspecific binding of protein to strain 602 and as a control for possible transfer of the ^{125}I label to cells of strain 602. Binding of ^{125}I-labeled bovine serum albumin to strain 602 was negligible under conditions identical to those used to demonstrate binding of IgG to strain 602.

Agglutination of IgG-opsonized strain 602 by monospecific antiserum to IgG heavy chains was used to confirm that IgG binds to the yeast and to establish that the IgG is bound directly at the yeast surface. Cells of strain 602 were incubated as described in Materials and Methods with various concentrations of pooled normal human IgG followed by antiserum to IgG. The results (Table 2) showed that very high concentrations of human IgG agglutinated strain 602 in the absence of antiserum to IgG. At lower concentrations of opsonizing IgG, opsonized cryptococci were agglutinated only by anti-IgG.

Opsonization of non-encapsulated *C. neoformans* by IgG. Having demonstrated that human IgG binds to the periphery of strain 602, we next determined whether this yeast-bound IgG could facilitate phagocytosis by macrophages. Phagocytosis of strain 602 by macrophages was determined in the presence of var-

TABLE 1. *Binding of IgG to C. neoformans strain 602*[a]

Expt no.	^{125}I-labeled IgG added (mg)	% Labeled IgG bound[b]
61	0.0198	0.14
61	0.0798	0.20
61	0.1600	0.28
62	0.0130	0.16
62	0.0260	0.12
62	0.0510	0.12
62	0.1030	0.12
62	0.4030	0.12
71	0.0705	0.35
71	0.2820	0.23
72	0.0705	0.28

[a] ^{125}I-labeled IgG was added to 10^7 cells of strain 602.

[b] Binding of IgG to strain 602 was determined as described in the text.

TABLE 2. *Agglutination of 602(IgG) by heavy chain-specific antiserum to human IgG*[a]

Anti-IgG[b]	Agglutination at the following concn of human IgG (μg/10^7 cells of 602):										
	1,250	625	312	156	78	40	20	10	5	2.5	—
Present	3+	4+	4+	4+	4+	4+	2+	1+	±	—	—
Absent	4+	3+	2+	±	—	—	—	—	—	—	—

[a] Agglutination was determined by both macroscopic and microscopic examination of cell settling patterns. Maximum observed agglutination was recorded as 4+. —, recorded as 4+.

[b] Heavy chain-specific antiserum to human IgG.

ious concentrations of normal human serum, heat-inactivated serum (56°C for 30 min), and purified human IgG. Concentrations of the three opsonins were adjusted to allow comparison of opsonizing activity in terms of IgG concentrations. The results of phagocytosis assays (Fig. 1) showed an expected dose dependence of the phagocytic index on each opsonin concentration. Examination of the data by analysis of variance showed no significant ($P = 0.05$) difference in effectiveness between any of the opsonin sources at any dose level studied. Thus, purified IgG accounted for all of the opsonization produced by normal human serum.

The above study showed that purified IgG alone could act as an opsonin, but the results did not indicate the nature of the interaction between 602(IgG) and the macrophage. Furthermore, the possibility must be considered that the diethylaminoethyl-cellulose-purified IgG might contain minute but effective amounts of some other opsonin. As a consequence, we sought confirmation for the role of IgG in phagocytosis by a technique that is independent of the purity of the opsonin source. Holland et al. (6) demonstrated that anti-macrophage IgG [AM(IgG)] specifically blocks Fc-mediated attachment and ingestion, but has little or no effect on non-Fc-mediated phagocytosis. These observations were confirmed by Griffin et al. (5) and extended to show that AM(IgG) does not block C3-mediated phagocytosis. We therefore attempted to determine whether AM(IgG) could block phagocytosis of IgG-opsonized 602. Erythrocytes opsonized with IgG were used as controls to demonstrate that the AM(IgG) did, indeed, block Fc-mediated phagocytosis. Macrophage monolayers were preincubated with 125 μg of AM(IgG) in HBSS, 125 μg of normal rabbit IgG[NR(IgG)] in HBSS, or HBSS alone for 60 min at 37°C. E(IgG), Formalin-treated erythrocytes, 602, or 602(IgG) were then added. Percent phagocytosis was determined after incubation at 37°C for 1 h. The results (Table 3) showed that: (i) AM(IgG) inhibited Fc-mediated phagocytosis of E(IgG); (ii) AM(IgG) had no effect on nonspecific phagocytosis of Formalin-treated sheep erythrocytes; (iii) AM(IgG) had no effect on nonspecific phagocytosis (non-IgG mediated) of strain 602; (iv) AM(IgG) reduced Fc-mediated phagocytosis of 602(IgG); and (v) normal rabbit IgG slightly reduced phagocytosis of E(IgG), but it had no effect on phagocytosis of 602(IgG). Increased phagocytosis of 602 in the presence of normal rabbit IgG is probably due to anti-cryptococcal antibody in rabbit serum. Control experiments (data not shown) showed that

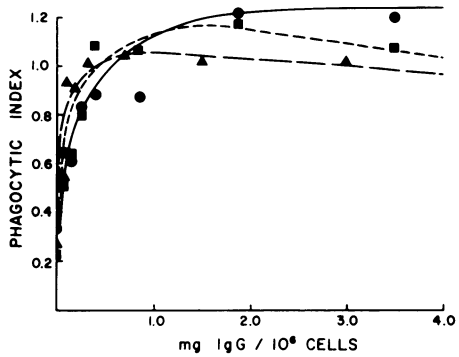

FIG. 1. *Phagocytosis of C. neoformans strain 602 in the presence of human serum (●), heat-inactivated human serum (■), and purified human IgG (▲).*

TABLE 3. *Inhibition of phagocytosis by anti-macrophage IgG*

Test particle[a]	Phagocytosis after macrophage treatment[b]		
	HBSS	NR(IgG)	AM(IgG)
E(IgG)	86 ± 3	67 ± 7	1.2 ± 1.2
Formalin-treated E	100 ± 0	100 ± 0	100 ± 0
602	25 ± 6	68 ± 10	27 ± 6
602(IgG)	62 ± 5	83 ± 6	41 ± 3

[a] E(IgG) were prepared by preincubation of 10^9 E with an equal volume of 1/800 7S anti-E. 602(IgG) were prepared by preincubation of 10^7 cells 602 with 1,250 μg of normal human IgG.

[b] Values indicate percent ± standard deviation. Macrophages were incubated for 1 h with HBSS, 125 μg of normal rabbit IgG, or 125 μg of anti-macrophage IgG before addition of the test particles.

AM(IgG) did not block phagocytosis of zymosan or latex particles.

The failure of AM(IgG) to reduce phagocytosis of 602(IgG) to levels seen with 602 alone was probably due to the level of opsonization of 602(IgG). Griffin et al. (5) showed that the blockade of Fc-mediated phagocytosis by AM(IgG) is quantitative, since inhibition can be overcome by increasing the density of opsonizing IgG on the test particle. Therefore, we investigated the effect of varying the amount of opsonizing IgG on the phagocytosis-inhibiting properties of AM(IgG). Cells of strain 602 were preincubated with various amounts of normal human IgG. These cells were then washed and added to macrophage monolayers that had been preincubated for 60 min at 37°C with HBSS or 125 μg of AM(IgG) in HBSS. Percent phagocytosis was determined after incubation at 37°C for 1 h. The results (Fig. 2) showed that AM(IgG) reduced phagocytosis of 602 opsonized with 312 μg of IgG/10^7 cells to the nonspecific background levels observed with 602 alone. As the amount of IgG/10^7 cells increased above 312 μg, AM(IgG) showed a decreased ability to block Fc-mediated phagocytosis. Thus, as with E(IgG) (5), the Fc-blocking activity of AM(IgG) is overcome by increasing the level of opsonization.

Inhibition of phagocytosis of 602(IgG) by AM(IgG) suggested that phagocytosis of 602(IgG) by macrophages is an Fc-dependent process. This suggestion was confirmed by use of $F(ab')_2$ fragments obtained by pepsin digestion of normal human IgG. Cells of strain 602 were preincubated with either purified normal human IgG or $F(ab')_2$ fragments of normal human IgG. These cells were then added to monolayers of macrophages, and percent phagocytosis was determined after incubation at 37°C for 1 h. The results (Table 4) showed that human IgG enhanced phagocytosis of strain 602, whereas phagocytosis of cells opsonized with $F(ab')_2$ fragments did not differ from unopsonized strain 602. Thus, data from this experiment and studies with AM(IgG) demonstrate a direct role for the Fc fragment in phagocytosis of 602(IgG).

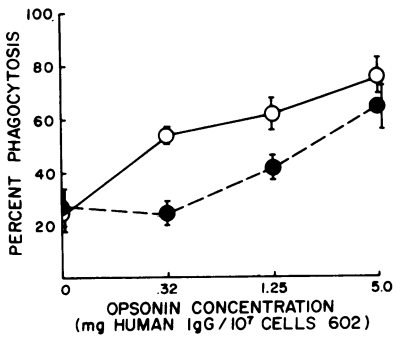

FIG. 2. *Effect of opsonin concentration on inhibition of phagocytosis of C. neoformans strain 602 by anti-macrophage IgG. Macrophages were preincubated with anti-macrophage IgG (125 μg/ml) (●) or HBSS (○) for 1 h at 37°C. 602(IgG) were added, and phagocytosis was determined after an additional 1 h of incubation at 37°C. Data are expressed as mean percent phagocytosis ± standard deviation.*

Role of IgG in attachment and ingestion. Since AM(IgG) blocks Fc-mediated attachment and ingestion (5), this reagent was used to determine the relative role of IgG in attachment and ingestion of 602(IgG). Macrophages were pretreated for 1 h at 4°C with AM(IgG), and 602(IgG) were added and incubated for an additional 2 h to assess the role of IgG in attachment. Alternatively, 602(IgG) were incubated at 4°C with macrophages for 2 h to allow attachment, but no phagocytosis to occur. AM(IgG) was added for an additional 1 h at 4°C, and the monolayers were incubated for 1 h at 37°C to determine the effects of AM(IgG) on ingestion. In each case, yeast cells were added to macrophages at a ratio of eight yeast cells per macrophage. Yeast cells were opsonized with 5,000 μg of normal human IgG/10^7 cells. Parallel experiments were done using E(IgG), since attachment and ingestion of E(IgG) are Fc mediated (5). The results (Table 5) showed that (i) the 4°C incubation allowed attachment of 602(IgG) to macrophages but inhibited ingestion; (ii) attached 602(IgG) were engulfed if the temperature was raised to 37°C; (iii) AM(IgG) substantially inhibited but did not eliminate attachment of 602(IgG) to macrophages; and (iv) AM(IgG) exerted only a minimal inhibition of engulfment of attached 602(IgG). AM(IgG) inhibited both attachment and ingestion of E(IgG) (data not shown).

The failure of AM(IgG) to effectively block ingestion of 602(IgG) could have been due to the high concentration of opsonizing IgG used in the experiment described above. If 602(IgG) had a greater density of opsonizing IgG than did E(IgG), it might have been possible to block ingestion of E(IgG) but not 602(IgG) with AM(IgG). Furthermore, the inability of

TABLE 4. *Opsonization of C. neoformans strain 602 by IgG and F(ab')₂ fragments of IgG*

Opsonin concn (μg/10^7 cells)	Phagocytosis[a]	
	IgG	$F(ab')_2$
5,000	75 ± 6	42 ± 4
1,000	74 ± 5	41 ± 1
None	40 ± 2	40 ± 2

[a] Values indicate percent ± standard deviation.

AM(IgG) to block ingestion of 602(IgG) might be due to some unknown artifact of the low-temperature incubation. Alternatively, the attachment phase of phagocytosis of 602(IgG) could be Fc mediated, whereas the ingestion phase might occur by a non-Fc-mediated process. The effect of 2-deoxy-D-glucose on phagocytosis of 602(IgG) was used to distinguish between these possible mechanisms. Michl et al. (12) showed that 2-deoxy-D-glucose had no effect on Fc-mediated attachment but inhibited Fc-mediated ingestion in a qualitative manner that was not influenced by the density of opsonin on the test particle surface. Accordingly, macrophage monolayers were incubated for 2 h at 37°C with MEM or MEM containing 50 mM 2-deoxy-D-glucose. E(IgG) or 602(IgG) were added and incubated for an additional 1 h. The macrophages were examined to determine percent attachment and percent ingestion. The results (Table 6) showed that both E(IgG) and 602(IgG) attached to macrophages in the presence of 2-deoxy-D-glucose; 2-deoxy-D-glucose inhibited ingestion of E(IgG) but had no significant ($P = 0.05$) effect on ingestion of 602(IgG). Thus, inhibition studies using AM(IgG) and 2-deoxy-D-glucose showed that attachment of 602(IgG) was greatly facilitated by opsonizing IgG, but the ingestion process was not Fc mediated.

TABLE 5. *Effects of anti-macrophage IgG on attachment and ingestion of IgG-opsonized C. neoformans strain 602*

Treatment	Attachment[a]	Ingestion[a]
602(4°C) → Wash[b]	59.1 ± 6.4	2.0 ± 1.6
AM(IgG) → 602(4°C) → Wash[c]	26.2 ± 5.7	0.9 ± 0.4
602(4°C) → Wash → 37°C[d]	0.9 ± 0.8	73.7 ± 4.1
602(4°C) → Wash → AM(IgG) → 37°C[e]	5.3 ± 1.5	60.0 ± 13.9

[a] Values indicate percent ± standard deviation.
[b] Macrophages were incubated with 602(IgG) at 4°C for 2 h, washed, and examined.
[c] Macrophages were preincubated with AM(IgG) (125 μg/ml) at 4°C for 1 h, 602(IgG) were added and incubated an additional 2 h, and the macrophages were washed and examined.
[d] Macrophages were incubated with 602(IgG) at 4°C for 2 h, washed, incubated 1 h at 37°C, and examined.
[e] Macrophages were incubated with 602(IgG) at 4°C for 2 h, washed, incubated with AM(IgG) (125 μg/ml) at 4°C for 1 h, and incubated at 37°C for 1 h.

TABLE 6. *Effect of 2-deoxy-D-glucose (2-dG) on phagocytosis of C. neoformans strain 602*[a]

Particle	Untreated		2-dG treated	
	Attachment	Ingestion	Attachment	Ingestion
602(IgG)[b]	1.0 ± 1.0	75 ± 8	1.0 ± 1.0	71 ± 4
E(IgG)[c]	11 ± 1	85 ± 6	69 ± 3	33 ± 7

[a] Values indicate percent ± standard deviation.
[b] Strain 602 opsonized with normal human IgG.
[c] Sheep erythrocytes opsonized with the IgG fraction of antiserum to sheep erythrocytes.

DISCUSSION

IgG is the principal, if not the sole, opsonin in human serum for phagocytosis of *C. neoformans* by normal mouse peritoneal macrophages. Furthermore, this opsonization involves a direct interaction between the opsonizing IgG and the macrophage via an Fc-mediated process. Support for these statements is derived from several lines of evidence. First, studies using ^{125}I-labeled IgG and agglutination of 602(IgG) by heavy chain-specific anti-IgG demonstrated that a portion of the IgG found in normal human serum binds to *C. neoformans*. Binding to the yeast is a necessary prerequisite if IgG is to function as an opsonin. Second, purified human IgG can account for all of the opsonizing activity found in normal serum. No differences were noted between whole human serum, heat-inactivated human serum, and purified IgG when the opsonizing activities of these reagents were compared at equivalent concentrations of IgG. Third, phagocytosis of 602(IgG) was blocked by anti-macrophage IgG. It could be argued that our human IgG preparation was contaminated by trace amounts of a highly opsonic serum protein other than IgG; however, AM(IgG) specifically blocks Fc-mediated phagocytosis at the macrophage membrane (5, 6). The action of AM(IgG) is, therefore, independent of the purity of the IgG. Finally, $F(ab')_2$ fragments of IgG lack opsonizing activity, suggesting that opsonization by IgG is Fc dependent.

Opsonizing IgG exerts its influence primarily during the attachment phase of phagocytosis. Attachment of opsonized cryptococci to macrophages was inhibited by blocking Fc-dependent processes with AM(IgG) or by pepsin digestion of opsonizing IgG. However, if 602(IgG) were allowed to attach to macrophages at 4°C, a condition permissive for attachment but not ingestion (5, 10), AM(IgG) could not block ingestion of 602(IgG) when the incubation temperature was increased to 37°C. The failure of AM(IgG) to block ingestion of 602(IgG) might be due to a high density of opsonizing IgG at the yeast surface that could not be blocked by AM(IgG) or to some unknown artifact inherent in the low-temperature incubation; however, ingestion of 602(IgG) was not blocked by 2-deoxy-D-glucose, a reagent that blocks Fc-mediated ingestion at 37°C by a mechanism that is independent of the density of opsonizing IgG (12).

Our results suggest that phagocytosis of *C.*

neoformans by normal mouse peritoneal macrophages involves a combination of two separate mechanisms. The first process is independent of serum opsonins. We have consistently observed phagocytosis of non-encapsulated cryptococci in the absence of any added serum opsonins (Fig. 1 and 2, Tables 3 and 4) (see Fig. 2 in ref. 10). Attachment and consequently ingestion are significantly lower without opsonization than attachment in the presence of serum opsonins. Nevertheless, there is some intrinsic surface property of non-encapsulated *C. neoformans* that permits low level phagocytosis by some nonspecific recognition mechanism. Since the ingestion phase of phagocytosis does not require opsonizing IgG, it is likely that this nonspecific mechanism is the mediator of ingestion. The nature of this nonspecific recognition is not known, but formation of divalent cation bridges (19) or attachment via van der Waals forces (14) have been suggested by some authors. Czop et al. (2) have recently reported that human monocytes ingest particulate activators of the alternative complement pathway in the absence of opsonizing protein. Since *C. neoformans* is a known activator of the alternative pathway (3), this as yet uncharacterized recognition mechanism may account for nonspecific recognition of *C. neoformans*. The second process operative in phagocytosis of non-encapsulated *C. neoformans* is IgG dependent. IgG stabilizes the attachment phase and permits more attachment than would occur in a given period of time by the nonspecific mechanism.

The serum opsonin requirements for phagocytosis of *C. neoformans* by normal mouse macrophages differ considerably from opsonins utilized by other phagocytic cells. Diamond et al. (3) reported that IgG facilitated phagocytosis of the yeast by neutrophils, but the IgG appeared to act indirectly as an activator of the complement pathway. Thioglycolate-activated (17) and glycogen-activated macrophages (13) required heat-labile opsonins, presumably complement fragments, for optimal phagocytosis of the yeast.

Earlier studies from our laboratory showed that cryptococcal polysaccharide inhibits the attachment rather than the ingestion phase of phagocytosis (10). Since the principal opsonizing action of IgG is seen during the attachment phase of phagocytosis, interference with or inhibition of the opsonizing action of IgG is one very likely mechanism by which cryptococcal polysaccharide might inhibit phagocytosis of the yeast.

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